Preliminary Report on the Study of the HUMET Composition's Anti-Clastogenic Effect in the Chromosomes of Human Peripheral Blood

The time of the study:

January 2 to 31, 1995

The subject of the study:

The numerical and structural changes of human peripheral blood lymphocytes due to the administration of HUMET®-R in various concentration.

Concentrations of HUMET:

We diluted 100, 200, 1000 and 2000 times the original stock solution in culture completed with RPMI-1640, 20% calf serum. We used 0.8 ml full[1] human peripheral blood and 0.2 ml Phytohemagglutinin (PHA) for the in vitro stimulation of the lymphocytes in the 10 ml agent. We had the cells grow through two cell cycles with HUMET being present and had it blocked with colcemide alkaloid (0.1 µl/ml) in the 52nd hour of propagation. Two hours later, following centrifugation, the cells' hypotonization and multiple fixation we prepared chromosome specimen with standard technology. The evaluation was accomplished according to WHO requirements with recording the numerical and structural changes. As controls we examined the chromosomes of untreated cells.

Based on the preliminary studies we have the following findings:

Control cells: 100 cells; 1% aberrant cell.

---

[1] ,

HUMET 100× dilution and HUMET 200× dilution: Both concentrations are non-physiological, since they cannot be found in such density in the organism. Nevertheless, in vitro growth could be detected, no chromosome aberration occurred in the received metaphase cells. The toxic effect of the substance should have been visible on the cells' morphology, their growth and in the alteration of the chromosomes. There was a single parameter smaller than among the controls, namely the PHA stimulation index. While the PHA stimulated lymphoblasts show approx. 70-80% growth in the control, the extremely high HUMET concentration reduces that approx. to its half.

Later, still not simulating physiological conditions, we investigated the effect of 1000× and 2000× dilution on the above parameters.

HUMET 1000× dilution and HUMET 2000× dilution: The cells display identical growth rate to the controls while the PHA stimulation shows identical blastogenesis indices. Similarly to the previous concentrations the substance's anti-clastogenic effect cannot be detected. The chromosomes show no numerical or structural alteration in the 200-200 examined cells.

Based on the above we can draw the following conclusion:

The poisonous, toxic or genotoxic effect of HUMET®-R together with its mutagenity in the lymphocytes of human peripheral blood can be clearly excluded, in spite of the fact that the dilutions employed were many hundred times stronger than the physiological concentration.

Opinion:

It is a subject of further studies whether clastogenes (chromosome-breaking agents) and HUMET applied together interact positively and whether more aberrations get induced by the mutagenic effect.

For this reason we wish to investigate the joint effect of ionising radiation, as a potential mutagene and clastogene, and various

concentrations of HUMET in the human peripheral blood test system.

Sarolta Gundy MD

Budapest, January 31, 1992

The Study of the Potential Anti-Clastogenic Effect of HUMET®-R in Human Peripheral Blood Lymphocytes

Budapest, 1992

by

Sarolta Gundy MD

Department of Human Genetics,

Medical Research Institute

Between December, 1990 and February 1991 we investigated the anti-clastogenic and/or mutagenic effects of HUMET®-R on the in vitro cultured lymphocytes of human peripheral blood.

It is well known that radiation or a good portion of chemicals may induce mutations in the cells of a living organism. If there is a DNA mutation in the nuclei of the cells transferred to the new cells, a new line of cells can be generated, which is a result of the given material's carcinogenic properties. Carcinogens, in turn, are potentially cancerous.

We know and employ several methods for monitoring the characteristics of carcinogenic and mutagenic material. Lately we have preferred citogenetic methods for, although the examination is expensive and requires sophisticated skills, it provides a fully realistic picture about the genotoxic (mutagenic) effect of the tested compound. The most hopeful among these is the chromosome analysis which looks at the numerical and structural aberrations of chromosomes and by the help of which a good part

of the mutations developed in the cells can be studied microscopically. We use the lymphocytes of leukocytes for chromosome analysis in the course of human in vivo and in vitro exposures. Among the advantages of the examination are that it measures on the level of human organism, and a few cubic centimetres of blood can provide several million cells. We prepare chromosome cultures by way of stimulating and propagating in vitro division of cells. Blood flows through all our organs, so if any mutagenic effect has reached the cells anywhere, then they carry the clastogenic effects of mutagenes as minuscule indicators.

However, in addition to in vivo examinations the detection of in vitro exposures also becomes possible. We treat the experimental system with special chemical or physical mutagenes before the incubation of the lymphocyte cell cultures, prior to cell division, in the so called G_0-phase. The damages caused by ionising radiation are able to induce aberrations in any stage irrespective of the cell's DNA synthesis phase. (The mutagenic effect of chemical substances can only be realised at certain points of the DNA cycle.)

Therefore, we studied the HUMET effect on the generally accepted mutagene, ionising radiation, in our model.

In order to study HUMET$_\bullet$-R's anti-clastogenic characteristics, first we needed to test the substance's mutagenity in the human lymphocyte system.

Detectable Chromosome Aberrations

We must keep in mind in case of the development of chromosome aberrations that there is a break or restructuring in the DNA double spiral constituting the chromosome. If the break is created in one arm of the chromosome, we call it chromatid deletion[2], if both arms are broken, we talk about chromatid break. Aberrations involving interchanges between two or more chromosomes result in di- or poly-centric chromosomes and rings. These latter ones

---

[2] loss

may almost have diagnostic value in detecting radiation effects, either in case of in vivo or in vitro induction.

Chemicals and UV radiation mainly causes chromatid changes, but ionising radiation create aberrations involving both arms of the chromosome (or of many chromosomes) in a multi-step mechanism.

Various types of aberrations are shown on Figures 1 to 4.

Numerical aberrations mean the detectability of cells displaying one or two alterations from the 46th chromosome. Though they are counted in in vitro examinations as well, we assign diagnostic value to them only if their frequency exceeds 5-7%.

In case of in vitro processes we evaluate the inter-relation of both the negative (untreated) and positive (mutagene treated) cells.

If the anti-Clastogenic effect of a substance is questionable, then the "second treatment" takes place simultaneously in the system of the treated cells.

We choose 2 Gy X-ray dosage in our study because it induces obvious quantities of aberrations with relatively little variance, the dosage can be controlled (see: radiation parameters), and mostly chromatid aberrations occur.

If HUMET$_a$-R proves to be a chemical mutagene, then in the course of mutagenic impact detection mostly chromatid aberrations must be revealed.

The Cultivation, Preparation and Evaluation of Chromosomes

Donors

We used heparin as an anticoagulant in the blood of two healthy women (20 and 24) and then treated in 10 ml maximum capacity media (RPMI-1640), which contained 0.8 ml blood either native or X-rayed with 2 Gy, the tested HUMET concentrations and Phytohemagglutinin M to prepare the cell division. We incubated the cells until the first division on 37°C and treat with colcemide

in the 48th hour of the propagation in order to stop the cell division. After that the freeing of the chromosomes from the cell membrane was done with conventional hypnotising, followed by 4-5 fold fixing. After draining the cell suspension we painted the cells with Giemsa solution and conducted a chromosome evaluation according to WHO provisions.

Results

We tested the mutagenic, on the irradiated cells the anti-Clastogenic, effect of various concentrations of HUMET$_{\bullet}$-R.

The test results of the mutagenic effects of the various HUMET concentrations are to be found in Table 1. We investigated 200-200 cells against the controls. It can be concluded that no chromosome aberrations were induced by any of the concentrations used, all of them substantially higher than the physiological solution, which fully excludes the chromosome level effect of mutations. (Finer mutations can be detected with the Ames Test.) Thus, HUMET is not Clastogenic even in such a high concentration.

The test results detecting the anti-Clastogenic efficiency can be found in Table 2. The result were calculated from aberration analysis following the non-normal distribution of 200 cells with the Poisson error of the average projected on a single cell, in accordance with international standards of evaluation.

Numeric aberrations were not detectable in any one of the cases. The proportion of chromatid breaks was not significant either.

The number of chromatid acentric fragments did not change with the applied HUMET concentrations. The di-centric ring chromosomes, developed by way of a complicated mechanism, revealed a decreasing tendency with the decrease of the concentrations. It seems that the chromosomes specific to irradiation effects reach the smallest value when 1 µl/ml final concentration was applied. The number of cells carrying the aberration decreased only in case of the 5 µl/ml concentration.

Since the number of aberrations within a single cell could be even higher than ten, the decreasing frequency of the occurrence of aberrant cells themselves in a dosage value is significant. In case of cell level in vitro tests the 5 µl/ml final concentration is most likely a critical one, whose effect may be further investigated. It can be suspected that the repairing capacity of the cells improves when effected by a radical bonding agent in in vitro systems. In case of 2 and 1 µl/ml treatments the currently available information is not sufficient to explain why the frequency of the aberrant cells returns to the level of the irradiated controls.

Conclusions

HUMET$_{®}$-R does not have mutagenic effect in the in vitro system of human peripheral blood lymphocytes. Concentrations much higher than the physiological level did not reveal Clastogenic effects, which excludes the substances genotoxicity.

Among the chromosome aberrations induced by 2 Gy the number of di-centric ring aberrations decreases together with the decrease in the HUMET concentrations, but a significantly lower value of aberrant cells is reached only in case of the 5 µl/ml concentration. These results suggest certain in vitro anti-clastogenic effects of HUMET$_{®}$-R. However, the mechanism (repair induction? scavenger or anti-oxidant effect) cannot be interpreted because of the inconsequencies in the results gathered so far.

Table 1

The Study of the Citogenetic Effects of Various HUMET$_{®}$-R Concentrations in Human Peripheral Blood Lymphocytes

HUMET concentration µl/ml	Aberrations of chromosomes [%]			
	Chromatid break	Chromosome break	Bicentric chromosome	Aberrant cell
Control	0	1	0	1
100	1	0	0	1
200	0	0	0	0
10	0	0	0	0
20	1	0	0	0

Number of cells evaluated in each concentration: 200-200 metaphase

Table 2

The Study of the Citogenetic Effects of Various HUMET$_{®}$-R Concentrations for 2 Gy (200 rad) X-ray Irradiation Induced in Human Peripheral Blood Lymphocytes

HUMET concentration µl/ml	Chromosomal aberrations (±SEM)			
	Chromatid break	Chromosome break	Bicentric ring	Aberrant cell
Control	0	0	0	0
Only 2 Gy	0.01±0.001	0.29±0.002	0.42±0.002	0.41±0.002
10+2 Gy	0.01±0.001	0.24±0.002	0.40±0.002	0.41±0.003
5+2 Gy	0.00	0.25±0.002	0.37±0.003	0.26±0.003
2+2 Gy	0.01±0.001	0.27±0.003	0.36±0.003	0.35±0.004
1+2 Gy	0.00	0.25±0.002	0.33±0.003	0.38±0.003

Number of cells evaluated: 600 cells
Control irradiated only
in each experiment: 200-200 metaphase
SEM: Standard Error of the Mean